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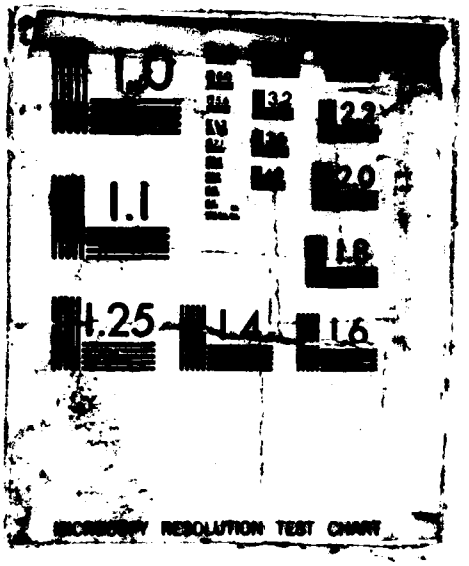
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Characterization of Rat Prothymocyte with Monoclonal Antibodies Recognizing Rat Lymphocyte Membrane Antigenic Determinants

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Utilizing the technique of fluorescence-activated cell sorting and monoclonal antibodies directed at rat membrane antigens, various subpopulations of Lewis bone marrow cells were isolated and subsequently transfused into sublethally irradiated, histocompatible NBr recipient rats by either intravenous or intrathymic inoculation. Recipient rats were sacrificed and cell suspensions from thymus and other lymphoid tissue were examined for the presence of the RT7.1 marker on Lewis thymus-derived lymphocytes by fluorescence-activated cell analysis. From these studies, the population of Lewis bone marrow cells that could reconstitute T cells in the NBr rats was found to be Ox-22 negative, Ox-7 positive, W3/13 positive, and Ox-18 positive. Further analysis characterized the prothymocyte as being Ox-7 upper 20% positive and W3/13 weakly positive. In addition this marrow cell population was able to protect lethally irradiated Lewis rats (9.5 Gy) in 30-day survival tests. These studies have indicated that the prothymocyte either has been derived from the Ox-22 negative, Ox-7 upper 20% positive, and W3/13 positive marrow cells or, like the hematopoietic stem cell, this cell has also been characterized by this phenotype.

INTRODUCTION

Recently, the rat hematopoietic stem cell has been isolated in our laboratory (1, 2). In the process of isolating and characterizing the hematopoietic stem cell population, several distinct cell populations within the marrow were definable by three monoclonal antibody reagents and immunofluorescent analysis. The three monoclonal antibodies, Ox-22, Ox-7, and W3/13, have been utilized in other laboratories (3–5) and in our laboratory to define marrow stem cell populations of immunohematopoietic lineages. Monoclonal antibody Ox-22 has been shown to recognize the high-molecular-weight form of the leukocyte common antigen and in the rat has been shown to more closely follow B-cell lineage (6). Ox-7 has been shown to recognize the rat Thy 1.1 antigen found on hematopoietic, T and B stem cells (7). W3/13 has been shown to recognize a leukocyte sialoglycoprotein that has been found on peripheral T cells, hematopoietic and T stem cells, and progenitor cells of the granulocytic series (8, 9).

Investigations in our laboratory (2) phenotypically defined the rat hematopoietic stem cell as Ox-22 negative, Ox-7 upper 20% positive, and W3/13 lower 50% positive,

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for when marrow cells with this phenotype were injected intravenously into lethally irradiated recipient rats, they were capable of generating hematopoietic spleen nodules (colony-forming unit spleen or CFU-S) and could protect the recipient rats from radiation-induced hematopoietic death. In addition, when these marrow cells were injected intravenously into sublethally irradiated histocompatible but allotypically disparate recipient rats, the development of donor-derived T lymphocytes was observed in the recipient donors. These studies indicated that the prothymocyte either was derived from the Ox-22 negative, Ox-7 upper 20% positive, and W3/13 positive marrow cells or; like the hematopoietic stem cell, was characterized by this phenotype.

In order to differentiate the prothymocyte from the hematopoietic stem cell, we have utilized a more sensitive assay system for studying the development of thymocytes from marrow cells (10). In the intrathymic adoptive assay system, marrow cells from donor rats are transferred directly into the thymus of sublethally irradiated histocompatible recipient rats, thereby providing the appropriate microenvironment for thymocyte development. These investigations on the isolation and characterization of the marrow prothymocyte population are reported in the present investigation.

MATERIALS AND METHODS

Animals. Viral antibody-free male NBr and Lewis rats were obtained from Charles River Labs (at the Kingston Production Facility, Stoneridge, NY) at 4 weeks of age. Experiments were performed at 6–8 weeks of age. Rats were euthanized with methofane.

Irradiation. Total-body irradiation of 7 or 9 (4 Gy/min) absorbed dosed was delivered with an opposing ^{60}Co radiation source. Irradiated rats were given 1.0 mg/ml tetracycline in their drinking water.

Reagents. Dulbecco's buffered saline (Gibco, Grand Island, NY) supplemented with either 10 or 2% heat-inactivated (56°C, 30 min) fetal calf serum (Hyclone Laboratory, Logan, UT) was used throughout and designated as 10 or 2% PBS. Phycoerythrins and conjugation reagents were purchased from Molecular Probes (Junction City, OR). Anesthetics utilized in this study were methofane (Pittman-Moore, Atlanta, GA), ketamine hydrochloride (Bristol Laboratories, Syracuse, NY), and acepromazine maleate (Fort Dodge Laboratories, Fort Dodge, IA). Tridak stepper was purchased from Indicon, Inc. (Brookfield Center, CT).

Antibody reagents. The following monoclonal antibodies were purchased from Accurate Chemicals, Inc. (Westbury, NY): Ox-7 HL, W3/13 HLK, W3/13 HLK–FITC, Ox-22, Ox-8, Ox-12, Ox-2, Ox-3, Ox-4, Ox-6, Ox-18, Ox-19, Ox-26, and W3/25. These monoclonal antibodies are characterized in Table 1. Monoclonal antibodies BC-84 and 8G6.1 have been characterized by Ely *et al.* (11). FITC–$F(ab')_2$ goat anti-rat Fc and FITC–$F(ab')_2$ goat anti-mouse Fc was purchased from Accurate Chemical. Preparation of phycobiliprotein–Fab' conjugates have been previously described (1, 2).

Cell preparation. Single-cell suspensions of bone marrow cells (BM) were obtained by flushing femurs with 4 ml of lysing buffer (ammonium-potassium chloride buffer, pH 7.0) through a syringe fitted with a 21-gauge needle, after which 6 ml of 10% PBS was added to the cell suspension. Cells were washed twice (1000g for 10 min) in 10%

PBS. Single-cell suspensions from thymus and other lymphoid tissue were prepared in 10% PBS by gently teasing cells from capsular material. Cell clumps were dispersed with a Pasteur pipet and after the debris had settled, cells were pipetted into test tubes. Cell suspensions were pelleted (1000*g* for 10 min) and the cell pellet was resuspended in 3 ml of lysing buffer for 3 min, at which time 7 ml of 10% PBS was added and cells were washed twice in 10% PBS (1000*g* for 10 min). Cells were then filtered through a 20.0-μm nylon mesh (Spectrum Medical Industries, Los Angeles, CA) to ensure a single-cell suspension. All procedures were performed at 4°C.

Labeling of nucleated cells. Labeling of cells with monoclonal antibodies has been previously described (1, 12). Briefly, cell suspensions were incubated with appropriate dilutions of monoclonal antibody reagent, incubated for 30 min at 4°C, washed two times in 10% PBS, and then resuspended in 2% PBS at 5×10^6 cells per milliliter for analysis and sorting.

Intrathymic adoptive transfer assay. Transfer of bone marrow cell populations was performed as previously described (10). Irradiated rats (7 Gy, 4 Gy/min) were anesthetized with a mixture of ketamine hydrochloride (1.20 mg/100 g body wt) and acepromazine maleate (4.4 mg/ml/100 g body wt). A midline incision was made in the skin over the thymic region and then a midline incision was made in the upper third of the sternum to expose the thymus. Cells were injected into the anterior lobes of the thymus using a 1-ml syringe fitted with a 28-gauge needle mounted on a Tridak stepper. After injection, the wound was closed with wound clips.

Intravenous adoptive transfer. Nucleated rat bone marrow cell populations in 1 ml of 2% PBS were injected intravenously (tail vein) into irradiated recipient rats using a 1-ml syringe fitted with a 21-gauge needle.

Cell sorting and analysis. Dual- and three-parameter flow cytometric measurements and sorting were made with the FACS-II equipped with the Consort 40 computer system (Becton–Dickinson FACS System, Mountain View, CA) as described previously (2). Briefly, the krypton laser was used as the primary laser and was tuned to the 530-nm spectral line for both phycoerythrin B and allophycocyanin excitation, while the argon laser (or delayed laser) was tuned to the 476-nm spectral line and was used for FITC excitation. The orange photomultiplier tube was shielded with a 575-nm bandpass filter (Becton–Dickinson). The red photomultiplier tube was shielded with a 660-nm bandpass filter (Diteric Optics, Hudson, MA), while the green photomultiplier tube was shielded with a 514-nm bandpass filter (Diteric Optics, Hudson, MA).

RESULTS

Analysis of Marrow Prothymocyte Population by Intrathymic Adoptive Transfer Assays

Characterization by single Mab reagents. Lewis bone marrow cells were labeled with monoclonal antibody reagents directed at antigenic determinants on rat cells. The Mabs utilized in this experiment have previously been shown to characterize rat marrow into distinct cell populations (1). A characterization of these Mabs may be found in Table 1. Nucleated marrow cells from Lewis rats (RT7.1) were labeled with Ox-7 (APC-Ox-7 Fab'), Ox-22 (PE-Ox-22 Fab'), or W3/13–FITC and sorted into posi-

TABLE 1

Monoclonal Antibodies Recognizing Determinants on Rat Lymphocyte Membranes

Monoclonal antibody	Antigenic determinant recognized	Cell population recognized
Ox-2	Glycoprotein on thymocyte membrane	B cells, neuronal endothelial cells, thymocytes, dendritic cells
Ox-3	Polymorphic determinant on rat Ia	B cells, some epithelial cells, dendritic cells
Ox-4	Polymorphic determinant on rat Ia	B cells, 20% of thymocytes
Ox-6	Monomorphic determinant on rat Ia	B cells, 20% of thymocytes
Ox-7	Rat Thy 1.1 antigen	Thymocytes, stem cells, neuronal cells, immature B cells
Ox-8	Glycoprotein determinant on thymocyte membranes	Cytotoxic, suppressor T cells, NK, thymocytes
Ox-12	Rat κ-chains	B cells
Ox-17	α-Chain rat Ia-antigen	B cells
Ox-18	Monomorphic determinant rat Class I MHC	Class I MHC (RT-1A)
Ox-19	Glycoprotein on thymocytes	Thymocytes, peripheral T cells
Ox-22	High-molecular-weight form of leukocyte common antigen	B cells, some T cells
W3/13	Sialoglycoprotein of rat thymocytes	Thymocytes, peripheral T cells, granulocytes
W3/25	Glycoprotein on helper T cells	Helper T cells, thymocytes

tive and negative fractions on a FACS-II. The sorted fractions were then transferred into irradiated NBr (RT7.2) recipient rats. (Fluorescent and sort profiles are shown in Fig. 1.) Eighteen days post-transfusion, the presence of donor thymocytes in recipi-

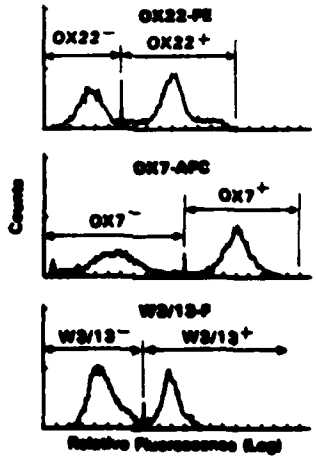

FIG. 1. FACS immunofluorescent profile of nucleated Lewis (RT7.1) marrow cells labeled with monoclonal antireagents Ox-22 Fab'-PE, Ox-7 Fab'-APC, or W3/13-FITC. Cells were sorted into positive and negative fractions on a FACS-II and then the sorted fractions were transferred intrathymically into irradiated NBr (RT7.2) recipient rats.

TABLE 2

Generation of Donor Thymocytes from Rat Marrow Populations Isolated by Single Monoclonal Antibody Reagents by the Intrathymic Adoptive Transfer Assay

Phenotype	Number donor cells transferred i.t.	Total number cells/thymus/10^7	% Donor thymocytes	Donor thymocytes per donor cell[a]
Normal BM	2×10^6	3.80 ± 1.95	79.50 ± 8.63	10.20 ± 3.77
	1×10^6	3.20 ± 1.79	31.90 ± 5.65	10.16 ± 3.17
Ox-22$^-$	1×10^6	4.00 ± 2.00	87.70 ± 9.36	34.73 ± 1.96
Ox-22$^+$	1×10^6	3.93 ± 1.98	<1	ND
Ox-7$^-$	1×10^6	3.47 ± 1.95	<1	ND
Ox-7$^+$	1×10^6	3.53 ± 1.88	69.90 ± 8.36	24.67 ± 1.57
W3/13$^-$	1×10^6	3.50 ± 1.87	<1	ND
W3/13$^+$	1×10^6	4.77 ± 2.18	87.13 ± 9.15	39.54 ± 1.99

Note. Irradiated (7.0 Gy) NBr (RT7.2) rats were injected i.t. with populations of purified Lewis (RT7.1) marrow cells. Eighteen days post-transfusion, donor origin thymocytes were quantitated by FACS immunofluorescent analysis. Results represent the means ± SD of three rats per group. Values of <1 indicate the absence of donor-derived thymocytes.

[a] The total number of donor thymocytes regenerated was divided by the total number of donor marrow cells transfused.

ent rats was determined by analyzing for Mab BC-84 positive cells on a FACS-II. BC-84 is a Mab that recognizes the RT7.1 alloantigen on donor thymocytes and T cells. The intrathymic transfer of donor marrow cell populations has delineated the marrow prothymocyte phenotype to be Ox-22 negative, Ox-7 positive, and W3/13 positive (Table 2).

As shown in Fig. 1, approximately 50% of the nucleated bone marrow cells are Ox-7 positive, 50% are W3/13 positive, and 50% are Ox-22 negative. Sorting with either of these three Mabs would yield an approximately twofold enrichment over unsorted marrow. When the number of cells transferred remained constant (1×10^6 cells), the percentage of donor thymocytes observed in recipients was found to have doubled.

Characterization based on Ox-7 positive cells. Preliminary studies in our laboratory indicated that the marrow prothymocyte resided in the upper 20% Ox-7 positive cell population. Therefore marrow cells were stained with Mab Ox-7 reagent and sorted for the upper 20% positive cell population, with the remainder of Ox-7 positive and negative cells collected together. Results (Table 3) of this experiment have shown donor thymocyte regeneration to result from marrow cells in the upper 20% Ox-7 positive region. By sorting for the upper 20% positive Ox-7 marrow cells (Table 3, last column), a 30-fold enrichment of the prothymocyte was obtained when the number of donor thymocytes regenerated in relation to the number of donor marrow cells transfused was calculated.

Characterization of marrow prothymocytes by three immunofluorescent parameters. From the preceding experiments the probable phenotype of the prothymocyte was inferred to be Ox-22 negative, Ox-7 upper 20% positive, and W3/13 positive.

TABLE 3

Generation of Donor Thymocytes from Purified Marrow Cells Using the Intrathymic Adoptive Assay System

Phenotype	Number donor cells transferred i.t.	Total no. cells/ thymus/10^7	% Donor thymocytes	Donor thymocytes per donor cell[a]
Normal BM	1×10^6	3.20 ± 1.79	31.90 ± 5.65	10.16 ± 3.17
Ox-7 up 20%$^+$	1×10^5	3.60 ± 1.90	98.10 ± 9.22	321.00 ± 17.52
Ox-7 < 20%$^+$	5×10^6	3.90 ± 1.97	<1	ND
Ox-22$^-$, Ox-7 up 20%$^+$	1.2×10^3	5.17 ± 2.27	65.33 ± 8.08	2.82 ± 1.68 $\times 10^3$
Remainder	5×10^6	3.20 ± 1.79	<1	ND

Note. Irradiated (7.0 Gy) NBr (RT7.2) rats were injected i.t. with populations of purified Lewis (RT7.1) marrow cells. Eighteen days post-transfusion, donor origin thymocytes were quantitated by FACS immunofluorescent analysis utilizing the Mab BC-84 which recognizes a RT7.1 T-cell alloantigen. Results represent the means ± SD of three rats per group. Values <1 indicate the absence of donor-derived thymocytes.

[a] The total number of donor thymocytes regenerated was divided by the total number of marrow cells transfused.

Therefore, Lewis marrow cells were labeled with these three Mab reagents and were then sorted into one fraction containing Ox-22 negative, Ox-7 upper 20% positive cells, and another fraction containing the remainder of the marrow cells (Fig. 2). Eighteen days postintrathymic transfer of these two fractions into irradiated NBr re-

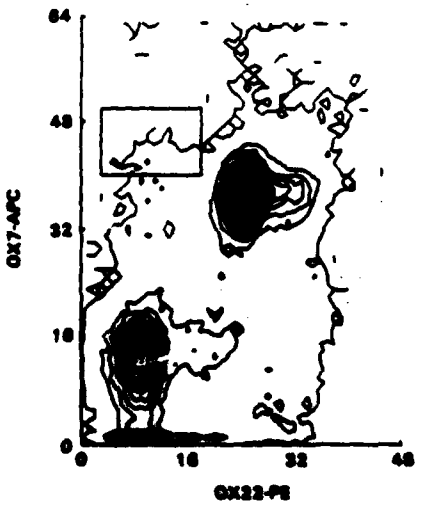

FIG. 2. Contour map of FACS-II immunofluorescent profile of nucleated Lewis marrow cells labeled with Ox-22 Fab'-PE and Ox-7 Fab'-APC. Boxed area denotes the prothymocyte population which is Ox-22 negative and Ox-7 upper 20% positive.

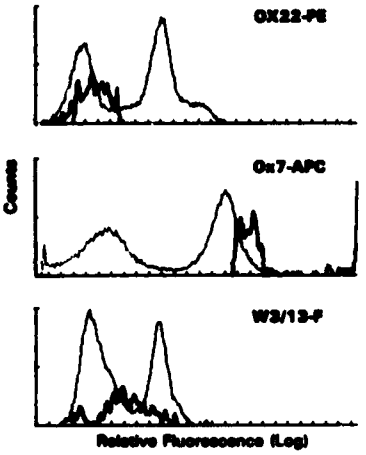

FIG. 3. FACS immunofluorescent profile of nucleated Lewis marrow cells labeled with Ox-22 Fab'-PE, Ox-7 Fab'-APC, and W3/13-FITC. Dotted lines represent profile of total marrow cells; solid lines represent profile of marrow cells with the Ox-22 negative, Ox-7 upper 20% positive phenotype.

cipient rats, donor thymocyte regeneration developed only in the Ox-22 negative, Ox-7 upper 20% positive population, confirming the phenotype of the prothymocyte. The results of these experiments are given in Table 3 (last column). When the number of purified prothymocytes transferred versus the total number of donor thymocytes regenerated was compared to that of whole marrow (versus the total number of donor thymocytes transferred), an enrichment of approximately 280-fold was obtained.

Analysis of the Ox-22 negative, Ox-7 upper 20% positive population in reference to W3/13 has shown these cells to be in the W3/13 lower 50% positive region. A profile showing the region of W3/13 positive cells contained within the Ox-22 negative and Ox-7 upper 20% positive population is given in Fig. 3.

Analysis of Marrow Prothymocyte by Intravenous Adoptive Transfer

Although intrathymic inoculation has been shown to be a more sensitive assay requiring less donor cells and providing the proper microenvironment for thymocyte development, it did not result in the establishment of permanent thymocyte chimeras (10). A specific criterion for a committed stem cell has included the characteristic that the stem cell be capable of replicating progeny-differentiated cells and of self-renewal (8, 13). In order to show that the marrow cell isolated in this study possessed the ability to produce progeny, the intravenous transfer assay was employed (14). Both unsorted marrow cells and the prothymocyte phenotype (Ox-22 negative, Ox-7 positive, and W3/13 lower 50% positive) cells isolated from Lewis donor rats were found to produce thymocytes in irradiated (9 Gy) NBr recipient rats 40 days post-transfusion (Table 4). As observed in i.t. transfer experiments, the number of thymocytes regenerated compared to the initial number of marrow cells transfused was significantly increased in the purified marrow population (Table 4). It should be noted, however, that in order to attain donor thymocyte regeneration, more cells were required when injected iv (3×10^6 marrow cells or 3×10^3 purified cells) than

TABLE 4

Generation of Donor Thymocytes from Purified Marrow Cells Using the Intravenous Adoptive Assay System

Phenotype	No. donor cells transferred	Total cells thymus $\times 10^7$	% Donor thymocytes	Donor cells thymus per donor cells transfused[a]
Normal BM	3×10^6	4.67 ± 2.16	94.13 ± 9.70	14.6 ± 1.21
Ox-22$^-$, Ox-7 up 20%$^+$	3×10^4	4.30 ± 2.07	88.10 ± 9.39	$1.27 \pm 1.13 \times 10^3$
Remainder BM	3×10^6	4.41 ± 2.7	<1	ND

Note. Irradiated (9.0 Gy) NBr (RT7.2) rats were injected intravenously with populations of purified Lewis (RT7.1) marrow cells. Eighteen days post-transfusion, donor origin thymocytes were quantitated by FACS immunofluorescent analysis utilizing the Mab BC-84 which recognizes the RT7.1 T-cell alloantigen. Results represent the means ± SD of three rats per group. Values of <1 indicate the absence of donor-derived thymocytes.

[a] The total number of donor thymocytes regenerated was divided by the total number of donor marrow cells transfused.

were required when injected i.t. (1×10^6 marrow cells or 1.2×10^3 purified cells), indicating that the i.t. assay system was a more sensitive assay system. In addition, donor-derived T cells were present in lymphoid tissues (spleen, peripheral lymph nodes, mesenteric lymph nodes, and Peyer's patches) of irradiated recipients at 6 weeks, 3 months, and 6 months post-transfusion, indicating that a stable T-lymphocyte chimera had been established from the marrow prothymocyte (Table 5). Analy-

TABLE 5

Generation of Donor T Lymphocytes in Recipient Lymphoid Tissue

Phenotype donor cells	No. donor cells transferred	Days post-transfusion	% Donor T cells in lymphoid tissue: Thymus	Spleen	Peripheral lymph nodes	Mesenteric lymph nodes	Peyer's patch
Normal BM	3×10^6	40	94.13 ± 9.70	21.20 ± 4.60	19.67 ± 4.43	15.03 ± 3.88	ND
		90	93.03 ± 9.65	26.97 ± 5.19	44.50 ± 6.67	49.07 ± 7.00	13.30 ± 3.65
		168	91.50 ± 9.57	33.20 ± 5.76	35.90 ± 5.99	41.13 ± 6.41	18.63 ± 4.32
Ox-22$^-$, Ox-7 up 20%$^+$	3×10^3	40	88.10 ± 9.39	27.03 ± 5.20	22.73 ± 4.77	22.80 ± 4.77	ND
		90	90.13 ± 9.49	39.23 ± 6.26	32.30 ± 4.77	41.80 ± 6.47	15.63 ± 3.95
		168	90.60 ± 9.52	37.47 ± 6.12	35.67 ± 5.97	37.47 ± 6.12	12.55 ± 3.54
Remainder	3×10^6	40	<1	<1	<1	<1	ND
		90	<1	<1	<1	<1	ND
		168	<1	<1	<1	<1	ND

Note. Irradiated (9.0 Gy) NBr (RT7.2) rats were injected i.t. with populations of purified Lewis (RT7.1) marrow cells. At 18, 90, and 120 days post-transfusion, lymphoid tissues were examined for the presence of donor T cells by FACS immunofluorescent analysis utilizing the Mab BC-84 which recognizes the RT7.1 T-cell alloantigen. Results represent the means ± SD of three rats per group. Values of <1 indicate the absence of donor T cells.

ses of lymphoid cells from Peyer's patches were not performed at 6 weeks because of the low numbers of cells in postirradiated tissue (12). Regeneration of Peyer's patches following 9 Gy has been found to be extremely slow.

Characterization of Prothymocyte Phenotype Marrow Cells with Other Monoclonal Antibody Reagents

From our investigations we have found that the marrow prothymocyte cell population is approximately 0.3% of total marrow cells. Single immunofluorescent analysis performed on 10^5 cells of marrow labeled with other Mabs, particularly if there are very few Mab-recognizable antigenic determinants on the prothymocyte, may not be sensitive enough to discriminate such labeling on whole-marrow analysis. Therefore, in order to determine if there were any antigenic sites on the prothymocyte recognizable by other Mabs, rat marrow was stained with various Mabs (Mabs characterized in Table 1) in addition to Ox-7 APC and Ox-22 PE. In order to obtain statistically significant numbers of prothymocytes, 300,000 cells were analyzed per Mab group. Analysis of data was made by observing which Mab reagents stained cells within the prothymocyte phenotype, i.e., gated on Ox-22 negative, Ox-7 upper 20% positive cells. The results, given in Fig. 4, noted that only three of the Mabs tested stained cells within this population—W3/13, Ox-19, and Ox-18. It should be noted that while this population stained brightly with Ox-18, the population stained dimly positive with W3/13 and Ox-19. As noted in Table 1, W3/13 and Ox-19 recognize antigenic determinants found predominantly on thymocytes and T lymphocytes while Ox-18 has been shown to recognize a MHC Class I antigen (8, 15).

DISCUSSION

Definition of the marrow prothymocyte population as well as other stem cell populations has been hampered by the inability to physically identify and isolate the putative cell populations and by the lack of specific assay systems by which the functional properties of the comitted cell may be determined. Definitive criteria for these stem cells would require that the cell must be committed to, but does not manifest, the genetic program for the progeny surface phenotype and that the cell can be induced to express this phenotype without intervening cell division (15). In addition, as a candidate stem cell, the cell would possess the capacity of self-renewal and production of progeny cell lineages that could reconstitute hematopoietic or lymphocytic populations in a recipient depleted of mature cell constituents (16–19). While many laboratories (20–22) have provided significant information characterizing immunohematopoietic stem cell populations within the marrow, the ability to characterize a single marrow stem cell population has proven more difficult.

By utilizing monoclonal antibody reagents directed at rat lymphocyte membrane determinants, the rat marrow hematopoietic stem cell was phenotypically characterized in this laboratory (2) as being Ox-22 negative, Ox-7 upper 20% positive, and W3/13 lower 50% positive. The concentration of cells in the marrow with this phenotype was calculated to be approximately 1500 to 3000 cells per million marrow cells or approximately 0.15 to 0.3% of the total marrow cell population. Although analysis of this population of cells by light scatter analysis revealed a fairly homogeneous population, by functional analysis the population contained three stem cell constitu-

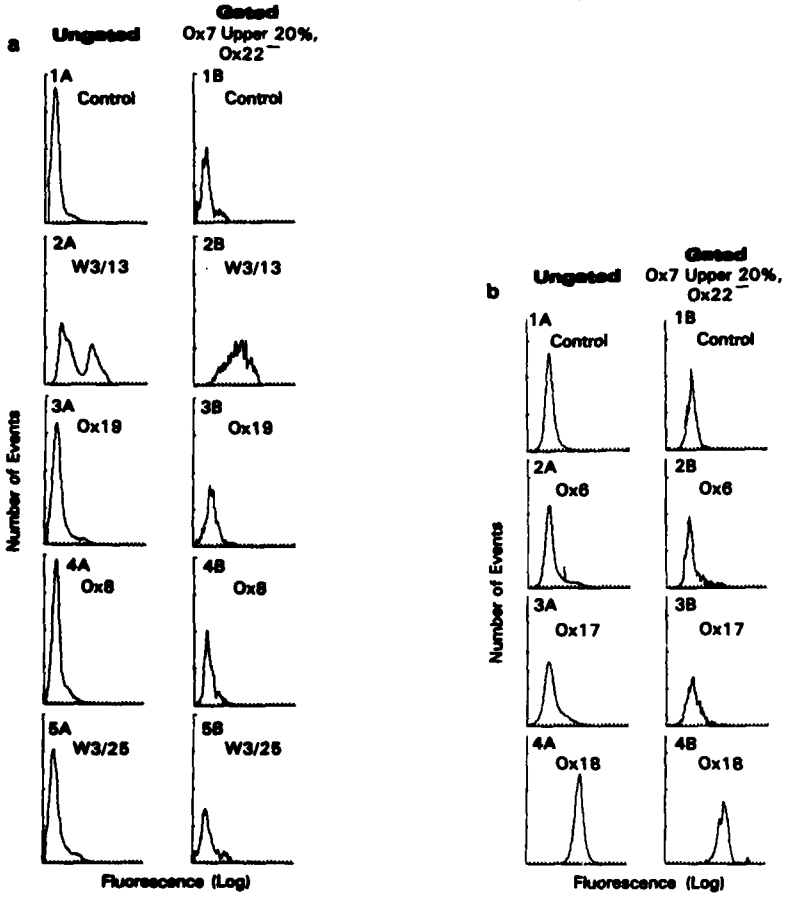

FIG. 4. FACS immunofluorescent profile of nucelated Lewis marrow cells labeled with Ox-22 Fab'-PE, Ox-7 Fab'-APC, and other monoclonal antibodies recognizing determinants on rat lymphocytes. Analysis shows fluorescent profile of the prothymocyte phenotype (Ox-22 negative, Ox-7 upper 20% positive) when labeled with Mabs recognizing rat T-cell membrane determinants (a) or with Mabs recognizing other lymphoid membrane determinants (b).

ents: the CFU-S, the hematopoietic stem cell, and the committed T stem cell or prothymocyte. The similarities between HSC and CFU-S have been discussed (2, 23–25), implying that maturational differences between the two stem cell compartments might result from external factors or accessory cells. In order to differentiate between the prothymocyte and the hematopoietic stem cell, populations of marrow cells were examined by two assay systems: (i) the intrathymic adoptive transfer assay system (10) that would show the ability of the presumptive marrow cell population to mature into phenotypically designated thymocytes; and (ii) the intravenous adoptive assay system (11) that would determine the proliferative capacity of the prothymocyte into T-lineage progeny.

As noted previously (10), the intrathymic adoptive transfer assay is significantly more sensitive than any other *in vivo* assay presently available for studying thymocyte

TABLE 6

Comparison of Intrathymic Adoptive Assay System with CFU-S and Radiation Survival Assay System for Analyzing Purification of Stem Cell Populations

Route of injection	Assay system	Number of cells transferred	Stem cell purification
iv	CFU-S	3000	100
iv	Irradiated recipient survival	3000	350
i.t.	Intrathymic adoptive transfer	1200	282

Note. Data from which these figures are compiled and calculated from experimentation performed in the present presentation (intrathymic adoptive transfer or i.t.) or in experimentation performed in Refs. (1) and (2) (colony forming unit-spleen or *in vivo* radiation survival).

regeneration from committed stem cell populations. Compared to methods utilizing intravenous transfer, 25- to 50-fold fewer donor cells are required by this method. In addition, when cells are injected directly into the thymus, the cells are provided with the proper microenvironment for thymopoiesis and are not subjected to extrathymic influences. Utilizing this highly sensitive assay procedure, prothymocyte populations were not observed in Ox-22 positive cells, Ox-7 negative cells, or W3/13 negative cells, and subsequently the phenotype of the marrow prothymocyte was found to be solely in the Ox-22 negative, Ox-7 upper 20% positive, W3/13 lower 50% positive fraction (Tables 2 and 3). These findings are in agreement with analysis by other investigators (4, 26) who have shown the rat prothymocyte population to be either in a W3/13 "dim" fraction or in an Ox-7 upper 10% positive fraction.

An essential point for the existence of a prothymocyte would require that the cell have the capability to home specifically to the thymus and give rise to cells in the T-cell lineage (27, 28). In our system, this question was answered by transferring donor marrow cells intravenously into sublethally irradiated rats and then following the regeneration of thymocytes and T lymphocytes in the recipients' lymphoid tissue. Both normal marrow and the Ox-22 negative, Ox-7 upper 20% phenotype cells were capable of homing to the thymus and then giving rise to T lymphocytes (Tables 4 and 5). Thymocytes were observed first in the thymus (18 days post-transfusion, unpublished data) and then T lymphocytes were observed in the spleen, peripheral lymph nodes, Peyer's patches, and mesenteric lymph nodes (6–24 weeks post-transfusion). Donor T lymphocytes could be detected in these lymphoid organs at least 6 months post-transfusion, indicating the formation of a stable chimeric animal.

From the present investigations performed in this laboratory, we have not been able to differentiate between the rat marrow hematopoietic stem cell and prothymocyte. Moreover, as shown in Table 6, measurements of the purification of the presumptive stem cell by intrathymic adoptive transfer or by 30-day survival assays have been similar and might indicate that the intrathymic adoptive transfer assay could preclude the need for radiation lethality assays. Further analyses on differentiating between these two stem cells and utilizing the intrathymic adoptive assay as an indicator for quantifying the number hematopoietic stem cells are being continued in this laboratory, although as discussed by Lemischka *et al.* (29), specific analysis may re-

quire genetically tagged individual stem cells in order to follow maturational stages of individual cells within this phenotypically defined compartment.

In an effort to differentiate between the two putative stem cells, we have analyzed the Ox-22 negative, Ox-7 upper 20% positive cell compartment for the presence of other antigenic markers. As shown in Fig. 4, monoclonal antibody W3/13 and two other monoclonal antibodies, Ox-18 and Ox-19, recognize antigenic determinants on this cell population. Monoclonal antibody Ox-18 recognized a rat MHC Class I determinant and, by immunofluorescent analysis, the stem cell apears to be brightly positive for the Class I determinant. Monoclonal antibodies W3/13 and Ox-19 both recognize determinants on thymocyte membranes. The cell population investigated has been shown to be W3/13 positive by Dyer and Hunt (4) as well as in this laboratory (1, 2), although definitive studies characterizing the amount and distribution of W3/13 on the stem cell population do need further clarification. By specific analysis of the stem cell population (Ox-22 negative, Ox-7 upper 20% positive cells) we have shown that these cells are labeled "dimly" with Ox-19. From our studies, we have reason to believe that this is not an artifact, but further investigations have been initiated in this laboratory to look more closely at this finding.

The characterization of the Ox-22 negative, Ox-7 upper 20% positive population with other monoclonal antibodies should provide a more complete profile of the stem cell population and, by understanding the purpose of the antigenic determinants identified, should lead to clarification on the maturation, physiology, and possible subtle differences between stem cells within this cell population. In addition, while implicating the relative closeness of the two stem cell populations (prothymocyte and hematopoietic stem cell), the finding that two monoclonal antibodies recognizing determinants on T lymphocytes have been found on the stem cell population should be carefully regarded in the practice of T-cell depletion of marrow prior to bone marrow transplantation, for depending upon the monoclonal antibody utilized in the T-cell depletion step, the hematopoietic stem cell might also be depleted. Further investigations utilizing these three monoclonal antibodies are being undertaken in order to differentiate between the hematopoietic stem cell and the prothymocyte.

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REFERENCES

1. McCarthy, K. F., Hale, M. L., and Fehnel, P. L., *Exp. Hematol.* **13**, 847, 1985.
2. McCarthy, K. F., Hale, M. L., and Fehnel, P. L., *Cytology*, in press.
3. Greiner, D. L., and Goldschneider, I., *J. Exp. Med.* **156**, 1448, 1982.
4. Dyer, M. J. S., and Hunt, S. V., *J. Exp. Med.* **154**, 1164, 1981.
5. Thierfelder, S., *Nature (London)* **269**, 691, 1977.
6. Spickett, G. P., Brandon, M. R., Mason, D. W., Williams, A. F., and Woollett, G. R., *J. Exp. Med.* **158**, 795, 1983.

7. Williams, A. F., and Gagnon, J., *Science* **216**, 696, 1982.
8. Brown, W. R., Barclay, A. N., Sunderland, C. A., and Williams, A. F., *Nature (London)* **289**, 456, 1981.
9. Williams, A. F., Galfre, G., and Milstein, C., *Cell* **12**, 663, 1977.
10. Goldschneider, I., Komsclies, K. L., and Greiner, D. L., *J.Exp.Med.* **163**, 1, 1986.
11. Ely, J. M., Greiner, D. L., Lubaroff, D. M., and Fitch, F. W., *J. Immunol.* **130**, 2798, 1983.
12. Hale, M. L., and McCarthy, K. F., *Radiat. Res.* **99**, 151, 1984.
13. Hunt, S. V., and Fowler, M. H., *Cell Tissue Kinet.* **14**, 445, 1981.
14. Greiner, D. L., Goldschneider, I., and Lubaroff, D. M., *Thymus* **6**, 181, 1984.
15. Dallman, M. J., Thomas, M. L., and Green, J. R., *Eur. J. Immunol.* **14**, 260, 1984.
16. Boyse, E. A., *Surg. Immunol. Res.* **4**, 6, 1985.
17. Schofield, R., and Lajtha, L., *Blood Cells* **9**, 467, 1983.
18. Mintz, B., Anthony, K., and Litwin, S., *Proc. Natl. Acad. Sci. USA* **81**, 2265, 1984.
19. Ross, E. A., Anderson, N., and Micklem, H. S., *J. Exp. Med.* **155**, 432, 1980.
20. Van Zandt, G., *J. Exp. Med.* **159**, 679, 1984.
21. Visser, J. W. M., Bauman, J. G. J., Mulder, A. A., Eliason, J. F., and DeLewin, A. M., *J. Exp. Med.* **159**, 1576, 1984.
22. Castagnola, C., Wisser, J. W. M., Boersma, W., and vanBekkum, D. W., *Stem Cells* **1**, 250, 1981.
23. Mueller-Sieburg, C. E., Whitlock, C. A., and Weissman, I. L., *Cell* **44**, 653, 1986.
24. Nakhata, T., Gross, A. J., and Ogawa, M. *J. Cell Physiol.* **113**, 455, 1982.
25. Potten, C. S., *In* "Stem Cells, Their Identification and Characterization" (C. Pattern, Ed.). Springer-Verlag, New York, 1983.
26. Wu, A. M., Till, J. E., Simonvovitch, L, and McCulloch, E. A., *J. Exp. Med.* **127**, 455, 1968.
27. Greiner, D. L. Goldschneider, I., and Barton, R. W., *J. Exp. Med.* **156**, 1448, 1982.
28. Kadish, J. L., and Basch, R. S., *J. Exp. Med.* **143**, 1082, 1976.
29. Lemischka, I. R., Raulet, D. H., and Mulligan, R. C., *Cell* **45**, 917, 1986.